Investigation into Semen Characteristics of Naturally-Fertile Pairs in Vitro and Their correlation with Fertility in Florida Sandhill Crane

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Abstract Semen characteristics of naturally fertile pairs and their correlation with fertility of eggs in Florida sandhill crane (*Grus canadensis pratensis*) were examined. Six pairs were used in this experiment, and all of them that had bred before were housed in open individual pens. These pairs were isolated physically each other but not visually and audibly. Semen was collected twice (Tuesday morning and Friday afternoon) from February 26 to June 4, 1993. The standard methods at Patuxent Wildlife Research Center were used collecting and evaluating the semen and spermatozoa, and incubating the eggs. There were statistically individual variations ($p<0.05$) about successful collecting attempts, semen volume, semen concentration, spermatozoon's motility, spermatozoon's number per collection, live spermatozoon's number, male's response to semen collection, and morphology of spermatozoa except the giant cell. There was a significantly ($p<0.05$, correlation coefficient was (0.73 or (1.00) negative correlation between fertility of eggs and the multiple value of semen (sperm) characteristics of naturally fertile pairs in Florida sandhill crane.

Key words: Florida sandhill crane, *Grus canadensis pratensis*, Semen (sperm) characteristic, Egg fertility

Introduction

In 1980, Thomas Lovejoy of the World Wildlife Fund stated that reduction in the biological diversity of the planet is the most basic issue of our time and that slowing this process of "biotic impoverishment" is a great challenge to the ingenuity of biologists. Extinction of a species represents the loss of a resource that has evolved through thousands, perhaps millions, of years of mutation and natural selection (Durrant, 1990).

As the number of birds' species threatened with extinction increase, agriculturists and conservationists have joined force in an effort to breed certain endangered bird in captivity to increase their numbers (Martins, 1975). However, because of severe space limitation in zoo, animal park, and the other organization concerned, this ideal genetic diversity will not likely be attainable for any of species we are attempting to save from extinction. The development and application of artificial insemination and semen cryopreservation techniques make them be possible that holding space requirements can be minimized and the number of species, that can be conserved through captive breeding, can be increased. It also makes it be possible that would effectively increase the genetic diversity of captive populations without altering or reducing the already limited free-ranging populations.

As the federal wildlife research organization, Patuxent Research Center has been doing research about the endangered Whooping Crane (*Grus americana*) and the Mississippi sandhill crane (*G. canadensis pulla*) for several decades. Every year we take semen samples from our endangered cranes, freeze the semen and add the frozen semen to the semen bank (gene pool). In earlier years, most our endangered cranes were artificially inseminated. But, with the lapsing of time, with the increasing of propagation generation, eventually, most pairs would produce fertile eggs without artificial insemination (naturally fertile). We would still need to collect semen from new males and add their semen to semen bank (gene pool).

There have been some people, Cooper (1958), Kamar et al. (1972 and 1984), Ansah et al. (1985), Brillard and Mcdaniel (1985), Cevil and Bakst (1985), Wishart (1985), Mcintgre et al. (1986), Cook et al. (1990), and Omprakash et al. (1992), have already studied the relationship between semen (sperm) quality and quantity and fertility of the eggs. However, as yet there has been any study on semen (sperm) properties of naturally fertile pairs and their correlation with fertility of eggs. We have known that semen collection from naturally fertile pairs does not interfere with production of fertile eggs (Gee et al., Unpublished paper), but we need to know whether we will be able to get relative better semen (sperm) quality and quantity from the naturally fertile pairs who have relative higher level fertility of eggs.

The main purpose of this paper is to identify the properties of semen (sperm) of naturally fertile Florida sandhill crane (*G. canadensis pratensis*) pairs and their correlation with fertility of eggs, e.g. Whether relative better semen (sperm) quality and quantity will be able to be gotten from the pairs who have relative higher level fertility of eggs.

Materials and Methods

Birds

Six Florida sandhill crane pairs were used in this experiment, and all of them that had bred before were house in open individual pens at Patuxent Wildlife Research Center, Laurel, Maryland. Their flights were been restricting. These pairs were isolated physically each other but not visually and audibly. On average, these male bird's ages were 7.2±2.0d (range from 4.0~10.0) years old, and female bird's ages were 9.4±2.4d (range from 5.0~15.0) years old. There had been, at least, two years breeding histories of these female cranes before were used in this experiment.

Semen collection

The semen was collected twice weekly (Tuesday morning and Friday afternoon) from February 26 to June 4, 1993. For different pairs, semen collection times (collecting attempts) were different. As for how many times of semen collection dine, they were depended on when the pairs would lay the last eggs of the third clutch, and that meant while the last eggs of the third clutch were been getting, the semen collection was been stopping from the pair.

Whenever possible, the same semen expert did the semen collection as operator, and the same two experts attended the semen collection as assistants. We used the methods and equipment described by Gee (1978a, 1978b, and 1993a) did the semen collection.

Bird's response to semen collection

Each time, while we were collecting semen, the birds of male were been ratting on a scale of 0-4 according to their response to semen collection. Slightly different combinations of this response would result in a "–" or "+" added to numerical score (Gee, 1993a). Each time, there were three experts giving the score, then, getting a mean value.

Volume

The volume of semen was measured with 1cc syringe immediately after semen collected, and fences were excluded. In case the semen volumes were too small to measure, they would be drawn into a syringe with 0.050 mL BPSE extender (Gee, 1993a).

Motility of spermatozoa

In the field, a small quantity of undiluted semen samples collected in a plain (non-nepariniged) microhematocrit tube (fill a 1-2 mm section of the tube). In laboratory, the motility of semen was examined within two minutes of placing it under the 10 times' objective of the microscope. Whenever possible, more than one field was focused. Each time, there were three experts examining the samples, and getting a mean from the score gave by the three people. Scoring was from 0~4, 0 corresponding no motile sperms, and 4 meaning more than 75% motile sperms (Gee, 1993a).

Concentration of spermatozoa

While samples were been examined in motility, concentrations of spermatozoa also were been examining, but the examining was only initial examining. Scores were given , like the responses of birds to semen collections, from 0~4, slightly different combinations of these concentrations would result in a "–" or "+" added to numerical scores. In laboratory, diluted semen samples were prepared dilution 1:100 or 1:200 with BPSE extender containing 1% Eosin and 10% Formaline. The samples scored from 0~3^{+} were diluted 1:100 and scored 4~4 were diluted 1:200, these could decrease the concentrations and make spermatozoa easy counted.

The numbers of spermatozoa were counted in four large corner squares (each containing 16 small squares) of an Improved Neubauer chamber (Hausser et al., 1967) under high power (430 times). Mean counts were obtained in the 2 chambers. Before counting the number of sperms, expel the first drops from the pipette and fill two sides of the chamber, and allowed 2~10 min (depending on the settling) for cells to settle.

Morphology of spermatozoa

In laboratory, the morphology of spermatozoa was examined microscopically in smears stained with Nigrosin and Eosin (Lake and Stewart, 1978). Each semen sample was made three slides. The following methods were used to make slides: one drop eosin was in corner of the slide, three drops nigrosin were directly beneath eosin, and one drop diluted semen was next to eosin; then, mixing them following the sequence: eosin with semen, former with nigrosin (Gee, 1993b). About 20 hours later, the sperms were evaluated under high power (430 times) microscope. Whenever possible, 300 spermatozoa and at least ten field throughout the whole slide were counted on each of three slides. Gee (1978a) identified six distinct cell types as follows: normal (N), bent (B), swollen (S), giant (G), droplet (DL) and dead (D). This time, we added the other (O) type (spermatozoa did

not belong to the types listed above). The proportions of normal) eosin-impermeable and abnormal (both eosin permeable and eosin-impermeable) spermatozoa appearing in a sample were estimated (Chaudhyri, 1988).

Fertile test

The eggs of each clutch were moved into incubator, immediately after the second egg laid, incubating them at the same time. The fertility of egg was estimated by candling with a bulb-light source, during days of 7, 21, and 28 of incubation. If necessary, the eggs would be broken for estimating fertile or not. For the eggs that could not be estimated fertile or infertile were given a name NDE (no detectable embryo).

Statistics

We used statistical methods (Steal et al., 1960, and lehner, 1979) testing the relationships between the average of semen (sperm) characteristics (which were correlation with fertility including motility, concentration, percent dead spermatozoa, percent abnormal spermatozoa, and the number of live spermatozoa per collection of total collecting times) and fertility of eggs. We also tested the relationship between the average of semen (sperm) properties, which were correlation with fertility and were from seven days to one day (24 h) before each egg laid, and the fertility of the eggs.

Results and Discussions

Semen and spermatozoa characteristics

Table 1 summarized the data, on collecting attempts, successful collecting attempts (SCA), mean semen quality and quantity per bird, which resulted from 6 pairs, 14 weeks, 28 times and total 105 collecting attempts. On average of all birds, collecting attempt, successful collection attempt (%), semen volume, sperm concentration, sperm motility, and male's response were 17.5, 80 %, 37 or 44 mm^3, 1.9 or 2.3 million/mm^3, 3.5 scale, and 3.2 scale, respectively. And also on average of all birds, normal sperm, bent sperm, giant sperm, swollen sperm, droplet sperm, dead sperm, other sperm, live sperm, abnormal sperm, sperm number per collection, and live sperm number were 58.1%, 14.1%, 0.9%, 5.2%, 9.2%, 11.4%, 1.1%, 88.6%, 30.3%, 116.6 or 141.9 million/time, and 105.5 or 128.2 million/time, respectively.

Table 1. List of collecting times, mean successfully collecting attempts, male's responses to semen collections and semen (sperm) properties each Florida sandhill cranes

Pen # of pair	R7	R30	R40	R44	S2	Y36
Collecting attempts	20	15	13	13	28	16
Successful collecting attempts (%)	70	100	62	92	68	88
Semen volume (μl)[a]	10±14	94±64	20±23	16±12	43±40	37±39
Semen volume (μl)[b]	14±15	94±64	32±21	18 ±11	63 ±32	42±38
Sperm concentration (million/(μl)[c]	1.5±2.0	3.3±2.4	1.1±1.4	2.4±1.9	2.1±3.2	1.1±1.3
Sperm concentration (million/(μl)[d]	2.1±2.1	3.3±2.4	1.8±1.5	2.6±1.9	3.0±3.5	1.2±1.3
Sperm motility (scale)	3.0±1.5	3.9±0.3	3.4±1.4	3.4±1.0	3.9±0.3	3.2±1.5
Normal sperm (%)	55.1±13.9	62.3±10.5	63.4±6.2	45.5±14.7	64.9±10.9	57.5±9.8
Bent sperm (%)	14.5±7.5	13.7±4.3	11.5±4.5	19.0±5.6	10.2±5.5	16.0±8.7
Giant sperm (%)	0.9±0.6	0.6±0.5	0.6±0.7	0.8±0.5	1.3±0.4	1.0±0.7
Swollen sperm (%)	4.6±2.5	3.7±2.4	4.4±1.5	4.0±2.0	10.4±11.9	3.9±1.4
Droplet sperm (%)	7.3±3.4	9.9±4.3	9.5±4.0	13.6±6.4	5.7±2.4	9.3±4.5
Dead sperm (%)	15.8±12.1	8.9±7.8	9.5±6.0	15.4±15.1	6.8±5.3	11.9±10.1
Other sperm (%)[e]	1.7±2.1	0.9±1.3	1.3±1.7	1.7±1.0	0.6±0.5	0.3±0.3
Live sperm (%)	84.2±12.1	91.1±7.8	90.5±6.0	84.7±15.1	93.2±5.3	88.1±10.1
Abnormal sperm (%)	29.1±9.2	28.7±7.3	26.0±4.4	39.1± 10.1	28.2±10.7	30.6±11.1
Sperm number (million/time)[f]	30.7±65.5	360.7±383.9	48.8±79.3	47.1±67.5	149.6±229.2	62.5±109.8
Sperm number(million/time) [g]	44.9±75.9	360.7±383.9	83.7±90.1	51.4±69.0	237.6±251.4	73.0±115.8
Response (scale)	3.2±0.8	3.9±0.3	2.6±1.4	3.2±1.1	2.7±1.5	3.7±0.5
Live sperm number (million/time)	25.9±55.2	328.5±350.0	44.2±71.7	39.8±57.1	139.4±213.6	55.1±96.7

a. Means: mean semen volume = total semen volume ÷ total collecting times.
b. Means: mean semen volume = total semen volume ÷ total successful collecting times.
c. Means: mean sperm concentration = total sperm concentration ÷ total successful collecting times.
d. Means: mean sperm concentration = total sperm concentration (total successful collecting times.
e. Means: mean morphology of sperm does not belong to the morphology listed above.
f. Means: mean number of sperm = total number of sperm ÷ total collecting times.
g. Means: mean number of sperm = total number of sperm ÷ total successful collecting times.

All of these semen (sperm) characteristics listed above and male's response to semen collection there were statistically significant individual variation ($p<0.05$) expect the morphology of the giant cell. Cooper (1958) and Gee (1978a) reported that semen volume and concentration were variable between birds within year.

Semen volume and morphology of sperm gotten by Gee(1971 and 1978a) from Greater sandhill crane (*G. c. tabida*) and the semen volume and morphology of sperm gotten from this experiment were listed in Table 2.

From Table 2, they could be known that although the results were from different subspecies and different breeding situation (single or pair), but the same collecting and evaluating methods, the results were same or nearly same. That meant that about the mean semen volume, percent normal, abnormal, dead and live spermatozoa there were not different between Greater sandhill crane and Florida sandhill crane, even though those cranes were in different breeding situations (single or pair).

Table 2. Comparison the semen volume and sperm morphology between Greater sandhill crane (single) and Florida sandhill crane (pair)

Species	GSC	FSC
Semen volume (mm^3)	30	37[a] or44[b]
Morphology (%)		
Normal	57.0±18.4	58.1±7.2
Bent	17.5±9.4	14.1±3.1
Giant	1.0±0.2	0.9±0.3
Swollen	6.0±4.3	5.2±2.6
Droplet	5.2±5.0	9.2±2.7
Abnormal	30.0	30.5±2.1
Dead	13.0	11.4±36
Live	87.0	88.6±3.6

a. Means: mean semen volume = total semen volume ÷ total collecting attempts.

b. Means: mean semen volume = total semen volume ÷ total successful collecting attempts.

Fertility and correlation

The number of good quality spermatozoa in a semen sample inseminated into a hen is one important factor determining the fertility level obtained by AI (Lake, 1983).Wishart (1985) reported that there was a non-linear relationship between number of spermatozoa and the probability of fertility. Lake (1989) also reported that the criteria ((motility(, (clumping of spermatozoa(and (morphologically abnormal spermatozoa(appeared to be suitable as predictors of individual male fertility. Omprakash et al. (1992) found that irrespective of dilution of semen, sperm concentration, motility and percentage of live sperms showed significantly positive correlation with fertilizing ability of semen, and percentage of abnormal sperms and MBRT value were negatively correlated with fertilizing ability of semen. Cooper (1958) presented that fertility was significantly correlated with percentage of dead spermatozoa ((0.89), motility (0.84), reduction time and live sperm density (0.55). According to the results gotten by those experts, and considering that more than one semen characteristics needed to be evaluated in order to predict the fertility of male (Lake, 1987), we listed fertility of eggs, and semen and spermatozoa characteristics which were correlation with fertility of eggs in Table 3. We were ratting on a scale of 1~4 or 1~5 or 1~6 for each characteristic of each bird according to that value was higher or lower and if there were two or three same numbers (same value was same scale). The sum was multiple value evaluating for the quality and quantity of semen (spermatozoa) of each bird. The relationship between multiple value and fertility was tested, the result was there was a significantly negative correlation (correlation coefficient was (0.73) between the multiple value of semen (spermatozoa) quality and quantity and the fertility of eggs.

There was a question needed to be explained as follows. From table 3, it could be known that the fertility of pair R7 was 60%. This male crane's left wing was injured and banded during after the female had finished first clutch and before she began to lay the third clutch. The three eggs of second clutch of pair R7, one was broken, the other two were infertile. We thought the infertile was correlation with the male's wing having been broken (affecting normal copulating). If the two eggs of infertile were excluded from counting the fertility, the fertility would be 100% for the bird. In turn, the correlation coefficient shown above would be increased 1.00.

Table 3. The relationship between the property of semen (sperm) and the fertility of egg

Pen# of pairs	R7	R30	R40	R44	S2	Y36
Fertility of egg (%)[a]	60	40	67	100	50	100
Sperm concentration (million/cmm)[b]	+3d	+6	+1	+5	+4	+1
Sperm motility (scale)	+1	+5	+3	+3	+5	+2
Dead sperm (%)	-6e	-2	-3	-5	-1	-4
Abnormal sperm (%)[c]	-4	-3	-1	-6	-2	-5
Live sperm number (million)	+1	+6	+3	+2	+5	+4
Sum	-5	+12	+3	-1	+11	-2

a. Fertility = fertile ÷ (fertile + infertile)

b. Mean sperm concentration = total sperm concentration ÷ total collecting attempts.

c. Abnormal sperm = total sperm – normal sperm – dead sperm

d. "+" means positive correlation with fertility of egg.

e. "–" means negative correlation with fertility of egg.

Since duration of sperm fertility was limited, not all

of semen samples gotten from successful collecting attempts were correlation with fertilizing eggs. Considering semen (sperm) quality and quantity were often variable within bird through breeding season (Gee, 1978a, and Gee et al., unpublished paper), it was the best way that the relationship between fertility and semen (sperm) characteristics was tested using semen characteristics of possible to fertilize eggs (within duration of spermatozoa's fertility). According to the shortest known sperm storage duration was six days (Park and Hardawick, 1987, and Birkhead, 1992), the properties of semen (sperm) of possible to fertilizing the eggs and the results (fertile or infertile) of eggs were statistically examined. Under the circumstance , which egg 3, 4, 5 laid by pair R7 were excluded, still there was a significantly negative correlation between the fertility of egg and the multiple value of semen (sperm) property.

Although we concluded that there was a significantly negative correlation between the semen (sperm) characteristics and fertility of egg, our results must be interpreted with caution for two reasons. First, the number of the pairs used in this experiment was only six, the samples were not big enough. Second, the methods evaluating the semen (sperm) were standard, but not the most superior. However, two methods (mean total semen characteristics and mean semen property from 7-1 days before the eggs laid) were used testing the relationship, and the same results had been gotten already, we though they could explain whether the results were accurate or not.

As the results shown above, relative better semen (sperm) quality and quantity in Vitro could be gotten from the naturally fertile pairs that there was a relative lower fertility. It could be explained from copulating behavior. Generally, the more the copulating was, the higher the fertility was, the less the semen was left. We got mean 94 mm^3 semen volume and 3.3 million/mm^3 spermatozoa's concentration, and at the same time we got 40% fertility from pair R30. Whereas the 16 mm^3 and 37 mm^3 semen volume and 2.4 and 1.1 million/mm^3 spermatozoa's concentration was gotten from pairs R44 and Y36, respectively, and in the same time the fertility of 100% and 100% were gotten from the two pairs.

The results presented above were identical with the requirement of captive breeding by artificial insemination. In general, male bird can not copulate or the copulating is not good enough, thus the eggs can not be fertilized or there are relative lower fertility. According to the results gotten from the experiment, semen collection done from this kind of birds can get relative higher quality of spermatozoa and relative higher quantity of semen. This is very important for captive breeding endangered species by artificial insemination.

While semen is being collected from new males for the purpose of storage semen for gene pool, it must be thought, according to the results shown above, which schedule is better.

Conclusions

There were statistically significant individual variations ($p<0.05$) about successful collecting attempt, means semen volume, mean spermatozoa concentration, mean spermatozoa motility, mean percent spermatozoa morphology (normal, bent, swollen, droplet, dead and other), mean percent live spermatozoa, mean spermatozoa number per collection and mean male's response to semen collection.

About semen volume and morphology of spermatozoa, there was not significant difference between different subspecies (Greater and Florida sandhill crane), and between different breeding situations (single or pair).

Semen collection from naturally fertile Florida sandhill crane pairs twice weekly, there was a significantly negative correlation ($p<0.05$, correlation coefficient was -0.73 or -1.00) between fertility of egg and the multiple value of mean semen and sperm characteristics (concentration, motility, percent dead sperm, abnormal sperm, and live sperm number), which gotten from all successful collecting attempts.

Semen collection from naturally fertile Florida sandhill crane pairs twice weekly, there was a significantly negative correlation ($p<0.05$, correlation coefficient was (0.73) between fertility of egg and the multiple value of mean semen (sperm) characteristics that were gotten from 7-1 days before the eggs laid.

To get relative higher spermatozoa's quality and semen quantity from naturally fertile Florida sandhill crane pairs, the semen collection can be done from the pairs that there are relative lower fertility.

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